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<b>13. ABSTRACT (Maximum 200 Words)</b> Investigation of basic factors involved in malignant transformation of the ovary has been hampered by the lack of an appropriate animal model. Most animals, with the exception of the domestic hen, do not spontaneously develop ovarian cancer. The use of two related genetic strains, which differ in spontaneous incidence of ovarian cancer may reveal an important difference between the two strains that could underlie the differential susceptibility to ovarian cancer. We have examined many hens of both strains and have observed that the marked difference in incidence between the strains has been maintained. We have characterized the tumors in terms of ovalbumin expression as an indication of site or origin. We have also examined the expression of markers in the tumors. Our second approach was to manipulate the rate of follicle development and ovulation to examine the effect of repetitive ovulation on incidence. This experiment was not possible so we have instead focused on hormones related to ovulation. We found that the C strain has higher circulating levels of progesterone as compared to the K strain. Finally, we are studying regulation of the tumor by investigating receptor expression in the tumors.				
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## INTRODUCTION

Investigation of basic factors involved in malignant transformation of the ovary has been hampered by the lack of an appropriate animal model. Most animals do not spontaneously develop ovarian cancer. This may be related to the fact that the usual condition of most wild and domestic animals is pregnancy and/or lactation. The exception is the domestic chicken, which has been demonstrated by several investigators to spontaneously develop ovarian cancer (Campbell, 1951; Wilson, 1958; Fredrickson, 1987). In this respect, as well as the fact that the chicken is a persistent ovulator (laying breeds ovulate almost daily), the chicken is similar to modern day women. That is, most women have 10-20 years of monthly ovulations prior to one or two pregnancies, with a subsequent 10-20 years of ovulations prior to menopause. The overall hypothesis of our DOD supported project is that the hen is an excellent model for human ovarian epithelial cell cancer. We will take a three-pronged approach in this project. First, we will compare differences in spontaneous incidence between the C and K strains of hens as they age and examine for pathological ovarian changes that may indicate site of origin of the tumors. Second, we will evaluate possible differences between the strains in response to reproductive manipulations highly correlated to incidence in women; and third, we will examine potential differences in cell signaling that may underlie the different incidence between the strains. It would be possible to conduct these experiments with a commercial strain of hens. We anticipate however, that use of the related C and K strains with different incidences of the disease, will provide a powerful tool that may reveal a potential marker of ovarian cancer.

## BODY

**Task 1. To characterize the incidence of spontaneous ovarian adenocarcinoma in 3-5 year old hens of the C and K strains and document histological changes in the ovary that may precede tumor formation (months 1-30)**

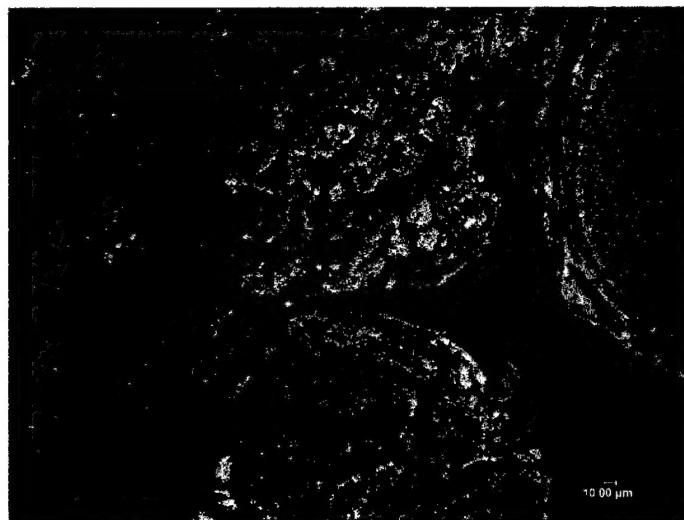
The overall goal of this task is to document the incidence of adenocarcinoma in hens of the C and K strain during their third, fourth and fifth year. In addition, we hope that frequent histological analysis of the ovary may reveal pre-cancer lesions. Incidence data for these strains was previously determined at approximately 2 years and we and others have seen a dramatic increase as hens age. Our data presented in our progress report from 2002 showed that the difference in incidence between the two strains has been maintained. We showed that 21% the C strain hens were diagnosed with ovarian cancer at approximately 3 years of age as compared to 0% in the K strain. We had also reported that we were interested in early signs of ovarian cancer and analyzed ovaries from hens not yet exhibiting overt ovarian cancer. As diagnosed by Dr. Prasad (our collaborating avian pathologist at the University of California at Davis), hens examined did not show overt ovarian cancer however, we did find hyperplasia and possible early neoplasia in the rete ovarii of one hen. Significantly, this was in a C strain hen at three years of age. We had previously shown that a number of hen ovarian tumors expressed ovalbumin. We have completed this study and show that all ovarian tumors removed from hens in which there was no oviductal involvement, as determined from gross examination and confirmed by Dr. Prasad (avian pathologist), expressed ovalbumin. This could be significant because it may indicate that ovarian tumor tissue is de-differentiated during the disease process. This may also indicate that the presence of ovalbumin is not diagnostic of oviductal origin of the tumor but may be correlated with the differentiated state of the tumor and therefore, may be a marker of the tumor in hens.

**TABLE 1**

Expression of ovalbumin in ovarian neoplasms of the domestic hen

Tissue	Ovalbumin positive
Ovarian neoplasms without oviductal involvement	10/10
Ovarian neoplasms with oviductal involvement	6/6
Normal oviduct	3/3
Normal ovary	0/9

The data in **Table 1** indicate that ovalbumin was expressed in all ovarian tumors, regardless of oviduct involvement. In addition, ovalbumin was not expressed in any of the normal ovaries examined. We have used a marker for cell proliferation (PCNA = proliferating cell nuclear antigen) in combination with the ovalbumin staining to examine the ovarian tumors. It is of interest to note (**Figure 1**) that many of the glandular areas expressing ovalbumin (green) are also areas of proliferation (red).



**Figure 1** This figure shows ovalbumin (green) and PCNA (red) staining of an ovarian tumor obtained from a C strain hen. (20x)

**Task 2.** **To manipulate the incidence of ovarian adenocarcinoma in the C and K strain of hens to test the effect of ovulation rate on a different genetic background.**

The aim in this study was to investigate the hypothesis that rupture and repair of the ovary during ovulatory events may be a factor in the development of ovarian cancer. Actively laying White Leghorn hens were administered pregnant mare's serum gonadotropin (PMSG) in an attempt to cause increased follicular development and superovulation. 150 I.U. of PMSG was administered intravenously for five to seven days in two separate trials. The effect of PMSG was determined by killing the hens and noting the size of the ovarian follicles, specifically those of pre-ovulatory size (>3mm.) We found that hens killed after five days of treatment showed evidence of increased follicular development as indicated by an abundance of medium-sized follicles as well as 2-3 pre-ovulatory sized follicles. This effect was increased in hens treated with PMSG for seven days. Such hens contained three to seven pre-ovulatory sized follicles, and numerous medium-sized follicles. Some hens also contained atretic follicles of pre-ovulatory size.

With the knowledge that PMSG was exerting the desired effect, we continued our investigation to determine whether we could induce multiple ovulations in the hens. Hens were

administered 400 $\mu$ g of luteinizing hormone (LH) 41 hours after their last PMSG injection (7 day course) and euthanized either eight or ten hours later. In the hens killed eight hours after administration of LH, we detected two ovulations (indicated by the presence of yolks in the reproductive tract as well as post-ovulatory follicles in the ovary) and noted that approximately three pre-ovulatory-sized follicles remained in the ovary. In the hens killed ten hours after LH injection, 3 yolks were found in the reproductive tract and three to five preovulatory-sized follicles remained in the ovary.

As controls, some hens received the same treatment (PMSG and LH) but were not killed. Our goal was to determine if we could induce additional multiple ovulations following the first. We found that these birds did not resume lay for quite some time after the initial LH injection. Some birds were administered additional LH and when killed, we found we were unable to induce ovulation in these birds.

We concluded that the effect of the PMSG in increasing follicular development (and, subsequently, taking the birds out of lay) was preventing further ovulation in these birds after the initial induced multiple ovulation. We attempted a shorter trial period of PMSG administration in the hopes that enough follicles would reach maturity and could be induced to ovulate, yet the effect was not so strong as to cause the bird to cease ovulating entirely. The hens were given PMSG for three days and then administered LH to induce multiple ovulation. Some birds in this treatment were killed and we observed that we were not able to induce multiple ovulation in these hens. Additionally, some of the hens not sacrificed ceased laying after only three days of PMSG treatment.

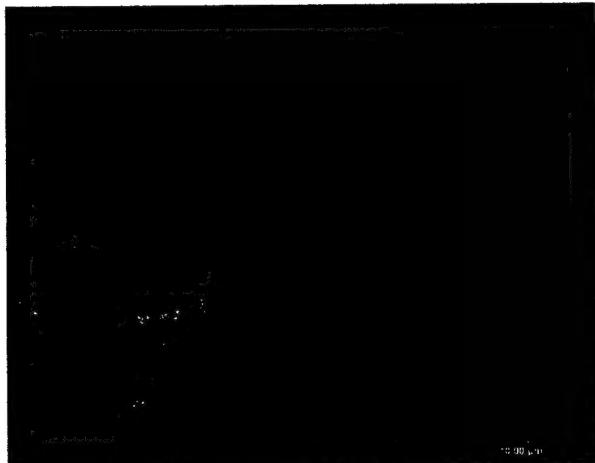
We concluded that the effect of PMSG varies greatly between the hens. We found it difficult to develop a treatment plan that caused enough follicular development to allow the induction of multiple ovulation yet did not take the birds out of lay. As the intent of our experiment was to increase the ovulation rate of the birds above the normal level, we concluded that the fact that many birds would stop laying as a result of PMSG administration would have adverse effects on our experimental aims.

As an alternate approach to this aim, we are studying the hormone profiles in the C and K strain hens. We hypothesized that there may be differences in steroid hormone secretion related to ovulation which could influence the incidence of ovarian adenocarcinoma between the two strains. In our recent study, we collected blood samples from hens at 1, 2 and 3 years of age. We used 10-12 hens of the C and K strain at each age. Hens were bled mid-cycle to avoid any preovulatory surges of hormones. Each hen was blood sampled twice (separated by 1 hour) to insure that baseline levels were found. These hens were matched for ovulation rate over the previous 6 month period. We found that the C strain had significantly higher plasma progesterone levels as compared to the K strain. In addition, there was no difference in either strain with respect to age. These results suggest that the ovarian steroid environment is correlated with susceptibility to ovarian cancer. We plan to assess estradiol concentration in these samples but have not yet done so.

**Task 3. To characterize the activity of the Activin/Smad signal transduction system in cell signaling in the normal ovarian epithelial layer and tumors from the C and K strain hens.**

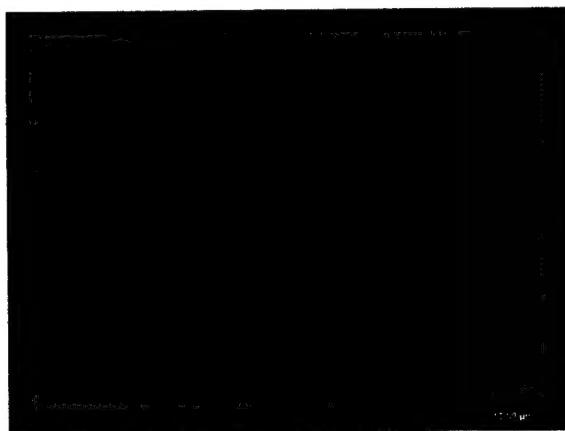
As stated in last year's report, we have developed a system for the culture of ovarian surface epithelial cells. More than 90% of human ovarian cancers are believed to arise from the single layer of epithelial cells that covers the ovarian surface. In order to begin the experiments for this task, it

was necessary to further characterize our culture system for the ovarian surface epithelial cells. We have been successful in culturing a pure preparation of ovarian surface epithelial cells but these cells are very difficult to grow. Since we know that these cells must be growing *in vivo*, we wanted to document the PCNA staining characteristics of the ovarian surface epithelial (OSE) layer. As shown in **Figure 2**, the OSE is stained positive for PCNA, indicating that cell proliferation is occurring in this layer.



**Figure 2** PCNA staining of OSE layer from a normal ovary. In this case, PCNA is stained green. 20x.

The expression of ovalbumin in ovarian tumors suggested that this protein might be regulated by steroids as ovalbumin is in the oviduct. Progesterone is an important steroid involved in the production of ovalbumin in the oviduct. For this reason, we decided to examine normal OSE as well as tumors for the expression of the progesterone receptor. We obtained an antibody which recognized the progesterone receptor in hens and used this antibody in immunocytochemistry. As shown in **Figure 3**, normal OSE (as well as other ovarian cell types) clearly express the progesterone receptor.



**Figure 3** Progesterone receptor (green) in the epithelial cell layer of a normal ovary. 20x

In order to study the possibility that progesterone receptor could be involved in the regulation of the tumor, we studied the expression of the progesterone receptor in tumor tissue. As seen in **Figure 4**, nuclear progesterone receptor (green) was observed in the tumor. Although the progesterone receptor was expressed in diverse cell types in the ovary, strong expression in glandular-like structures was consistent a role in regulation of ovalbumin secretion.



**Figure 4** Progesterone receptor in a hen ovarian tumor showing nuclear staining in glands (green). Red staining is autofluorescence from red blood cells. 20x

## KEY RESEARCH ACCOMPLISHMENTS

1. We have continued to accumulate C and K strain hens of various ages and have evaluated the age-related disease process in the hens. Tumors as well as normal ovaries have been examined in both C and K strain hens at selected intervals. Our data indicate that expression of ovalbumin is not indicative of oviductal origin of the tumors. We would conclude that tumors originating in the ovary de-differentiate during the disease process and thereby express ovalbumin.
2. Although it was not possible to effectively increase the ovulation rate in the two strains of hens, we found that the C strain hens had significantly higher circulating progesterone levels as compared to the K strain. There was no difference with respect to age of the hen and average laying rate.
3. We have characterized the expression of ovalbumin and progesterone receptor in normal ovaries as well as ovarian tumors from hens. Ovalbumin is not expressed in normal ovarian tissue. Expression of ovalbumin in tumor-containing ovaries occurs in regions of the ovary that are actively proliferating as indicated by PCNA.

## REPORTABLE OUTCOMES

- 1) Giles, J.R., Shivaprasad, H.L. and P.A. Johnson. Expression of Ovalbumin in Ovarian Tumors of the Domestic Hen. Biology of Reproduction **68** (Suppl. 1):236, 2003. (Abstr.)
- 2) Giles, J.R. Olson, L.M. and P. A. Johnson. The occurrence of ovarian cancer in the hen; validation and characterization. In prep., to be submitted to JNCI.
- 3) Giles, J.R., Shivaprasad, H.L. and P.A. Johnson. Expression and Regulation of Ovalbumin Production in Ovarian Tumors of the Hen. In prep., to be submitted to Gynecologic Oncology.

## CONCLUSIONS

This project is important because the hen spontaneously develops ovarian adenocarcinoma and therefore, questions related to etiology can be examined. This work is innovative because although previous workers have described ovarian adenocarcinoma in the hen, they have not attempted to study the regulation nor characterize the cell types involved. In addition, the use of two related genetic strains which differ in spontaneous incidence of ovarian cancer may reveal an important difference between the two strains that could underlie the differential susceptibility to ovarian cancer.

Our initial studies were directed at comparing ovarian cytology in normal hens and those with ovarian adenocarcinoma. We have examined many hens of both strains and have observed that the marked difference in incidence between the strains has been maintained. We have characterized the tumors in terms of ovalbumin expression as an indication of site of origin. We have also examined the expression of markers in the tumors. Our second approach was to manipulate the rate of follicle development and ovulation to examine the effect of repetitive ovulation on incidence. This experiment was not possible so we have instead focused on hormones related to ovulation. We found that the C strain has higher circulating levels of progesterone as compared to the K strain. Finally, we are studying regulation of the tumor by investigating receptor expression in the tumors.

The main cause of the lethality of ovarian cancer is the fact that it is usually diagnosed at an advanced stage. The availability of an animal model which spontaneously develops ovarian cancer (unlike most other animal models) would enhance the chance of finding a marker for early diagnosis. Knowledge about the etiology of ovarian cancer may help in the design of more optimal treatments. In addition, an animal model would permit the testing of pharmaceuticals that may decrease the growth of this cancer. Characterization of the two genetic strains may permit the identification of potential tumor markers.

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**Title** EXPRESSION OF OVALBUMIN IN OVARIAN TUMORS OF THE DOMESTIC HEN

**Authors** James R. Giles PhD<sup>1</sup>, H. L. Shivaprasad BVSc, PhD<sup>2</sup> and Patricia A. Johnson PhD<sup>1</sup>

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**Abstract** The lifetime risk of ovarian adenocarcinoma for a young woman is approximately 1.5% and it is the most lethal of the gynecological malignancies. The etiology and early events in ovarian carcinogenesis are poorly understood. Ovarian tumors are rarely observed in most species with the exception of the domestic hen, which like the human spontaneously develops ovarian neoplasms. Human ovarian adenocarcinoma is thought to arise from the modified mesothelial cell layer overlying the ovary. The origin of ovarian neoplasms in the hen has not been determined because the oviduct, the peritoneum and sometimes the pancreas are often involved and it is therefore difficult to determine the primary site of the tumor. We hypothesized that ovarian tumors without oviductal involvement would not express the oviductal protein albumen, the major protein found in the magnum of the hen's oviduct. On the basis of gross visual exam, tissues were removed from hens determined to have ovarian tumors without (n=10) or with (n=6) oviductal lesions. The tissue samples were fixed in 10% neutral buffered formalin,

processed, embedded in paraffin, sectioned and stained with haematoxylin and eosin. Ovarian tumors and other peritoneal lesions were evaluated histologically. Paraffin sections of ovarian tissue were deparaffinized and incubated in pronase solution for antigen retrieval. Sections were incubated with rabbit antiserum raised against chicken ovalbumin. The second antibody was a fluorescein conjugated goat anti-rabbit IgG. The majority of hens (9/10) with ovarian adenocarcinoma without oviductal involvement were positive for ovalbumin in the ovary. Furthermore, ovalbumin was detected in all hens (n=6) with tumors on the ovary and oviduct. Ovary sections from normal hens (n=9) were negative and oviductal sections from normal hens (n=3) were positive. Finally, ovalbumin expression in ovarian sections was often localized in quite discrete patches. The presence of ovalbumin in the majority of ovarian tumors in the absence of any oviductal involvement could suggest that ovarian tumors de-differentiate during the disease process. We cannot eliminate the possibility of micro-metastases from the oviduct but the lack of any defined oviductal tumor suggests that the oviduct may not be the primary site of origin in most ovarian adenocarcinoma.

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